Biotech: Principles & processes

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* Introduction

- Biotechnology
→ Techiques of using living organism or enzyme from organism for products & services.

- Old/Traditional biotech
→ Techiques of using complete organism like for making curd (lactobacillus), Idli & Dosa (yeast), wine etc.

- Modern biotech
→ Techiques of using GMO (genetically - modified organism) for products & service

e.g. IVF, gene therapy, vaccine, test tube baby etc.

- The European federation of Biotechnology (EFB) has given a definition of biotech that encompasses both traditional view and modern molecular biotechnology. The definition given by EFB is as follows: The integration of natural science and organism, cells, parts thereof, and molecular analogues for products and services.

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- Molecular analogues
  ↳ oligonucleotide (DNA/RNA), insulin etc made in lab that mimics biological products.

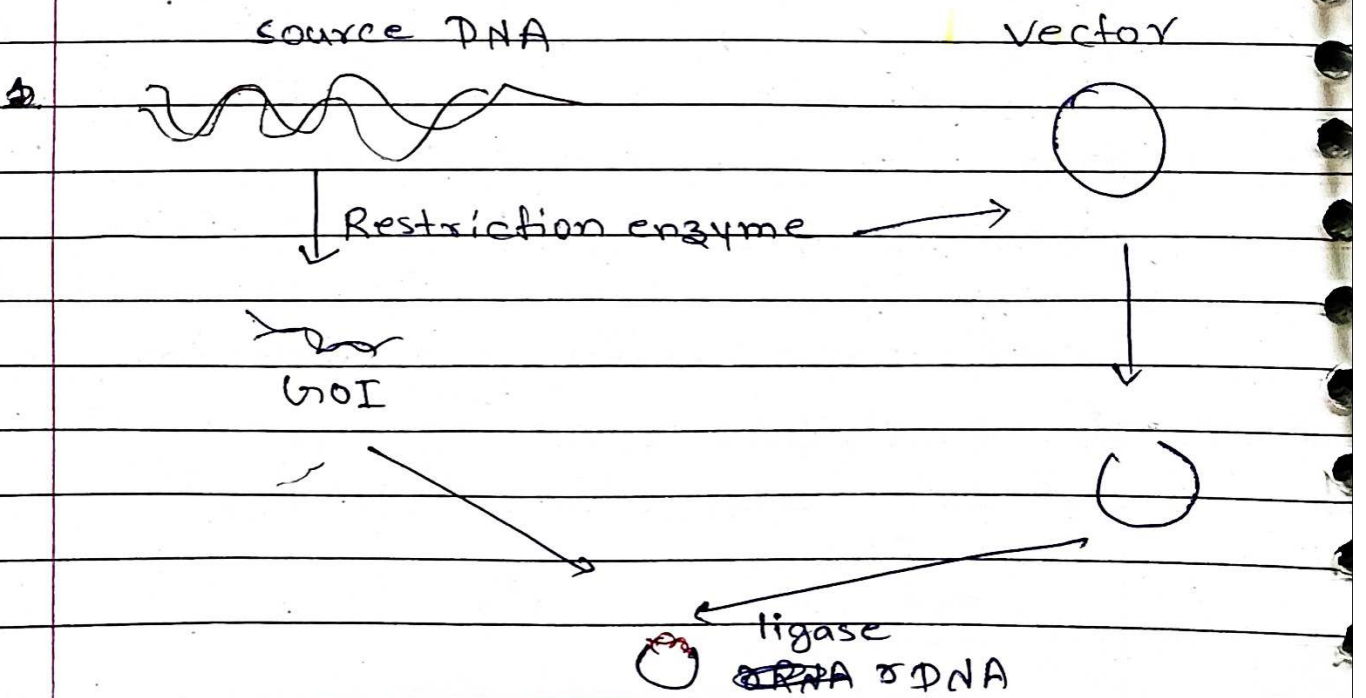
* Principle of Biotech

2 core techniques that enabled the modern biotech.

1. Genetic engineering
2. Bioprocess engineering

* Genetic engineering

- Here we do :-
  (i) Construction of rDNA (recombinant DNA)
  (ii) Gene tranfer
  (iii) Gene cloning



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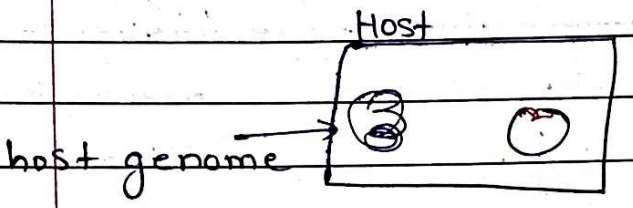
rDNA
∫ introduce in host



rDNA multiple, express in host

Tools of recombinant DNA Technology

(1) Lytic enzyme

→ Enzyme that open cell, so that DNA can be extracted for further process.

Cellulose ——→ for plant cell
lysozyme ——→ for bacterial cell
pectinase or chitinase → for fungi

(2) Restriction enzyme

(1) Discovery in 1963, 2 enzyme were discovered in bacteria.

∫↓

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When bacteriophage attack bacteria (E.coli)

↓

2 enzymes play role (defense mechanism of bacteria)

1st enzyme add methyl group to own genome

↓

Methylation cause protection of own DNA

2nd enzyme cleave foreign genome

↓

Restriction Endonuclease enzyme

- Restriction enzyme is named so because it restrict the growth of foreign DNA
- 1st restriction enzyme discovered → Hind II (characterization 5 year later)
- More than 900 restriction enzymes have been isolated from 230 strains of bacteria
- Restriction enzyme belong to Nuclease family (break phosphodiester bond)

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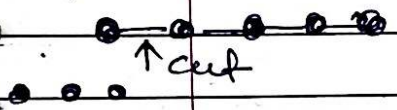
Nuclease

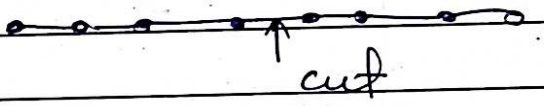
Exonuclease

→ cut at terminal or end
→ chop nucleotide from end



Endonuclease

→ cut in b/w or within



Endonuclease

Specific

→ Specific in nature
→ cut at or near recognition sequence
→ It is of 2 types

Type-I

→ cleave + methylation both property

Type-II

→ only cut property
→ cut at recognition site
→ require mg^{2+} (no ATP)
→ mostly use in Biotech

non-specific

→ non-specific in nature
→ cut anywhere within DNA sequence

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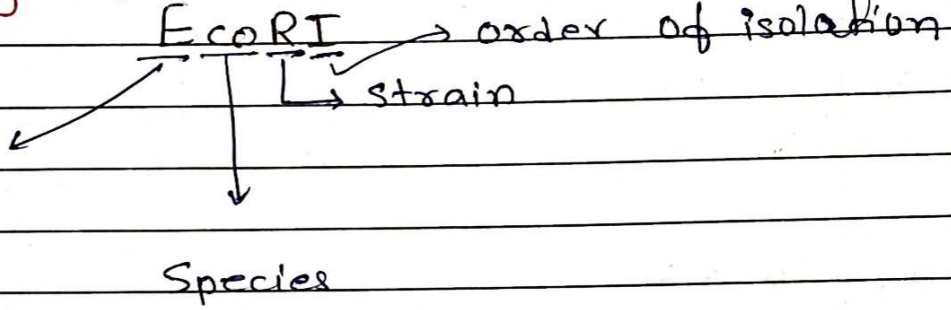
Type-I
→ Cut far from recognition site.
→ require ATP and mg^{2+}
→ rarely used in biotech

- daming of Restriction enzyme



Restriction enzyme	Source
(a) BamHI	Bacillus amyloliquefacien H
(b) EcoRI	Escherichia coli RY13
(c) EcoRV	strain of E. coli
(d) Sal I	Streptomyces albus
(e) HindII	Haemophilus influenzae Rd

- Restriction enzyme cut at restriction site

- Recognition sequence is the sequence of base pair where Restriction enzyme bind.

- Polindromes : English words which spell same in forward and B as well as reverse direction

e.g. $\overrightarrow{MADAM}_{\leftarrow}$, $\overrightarrow{MOM}_{\leftarrow}$

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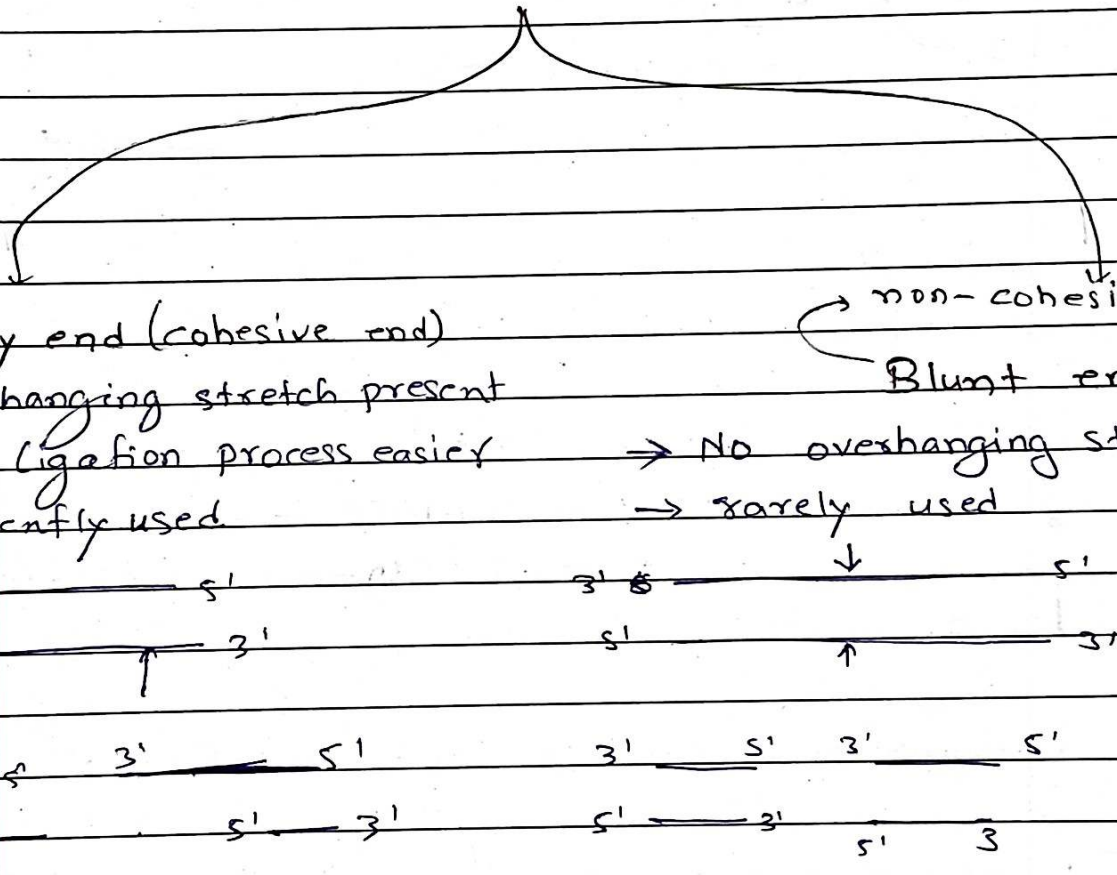
- Palindromic sequence ÷ Sequence of DNA which read same when orientation is kept same

3' — GAATTC — 5' → 5' to 3' (CTTAAG)

5' — CTTAAG — 3' → 3' to 5' (GAATTC)

- Restriction enzyme produce two types of end



Note ÷ Always we used same restriction enzyme to treat our DNA and Vector. So that same type of sticky end is produced and ligation will be easy.

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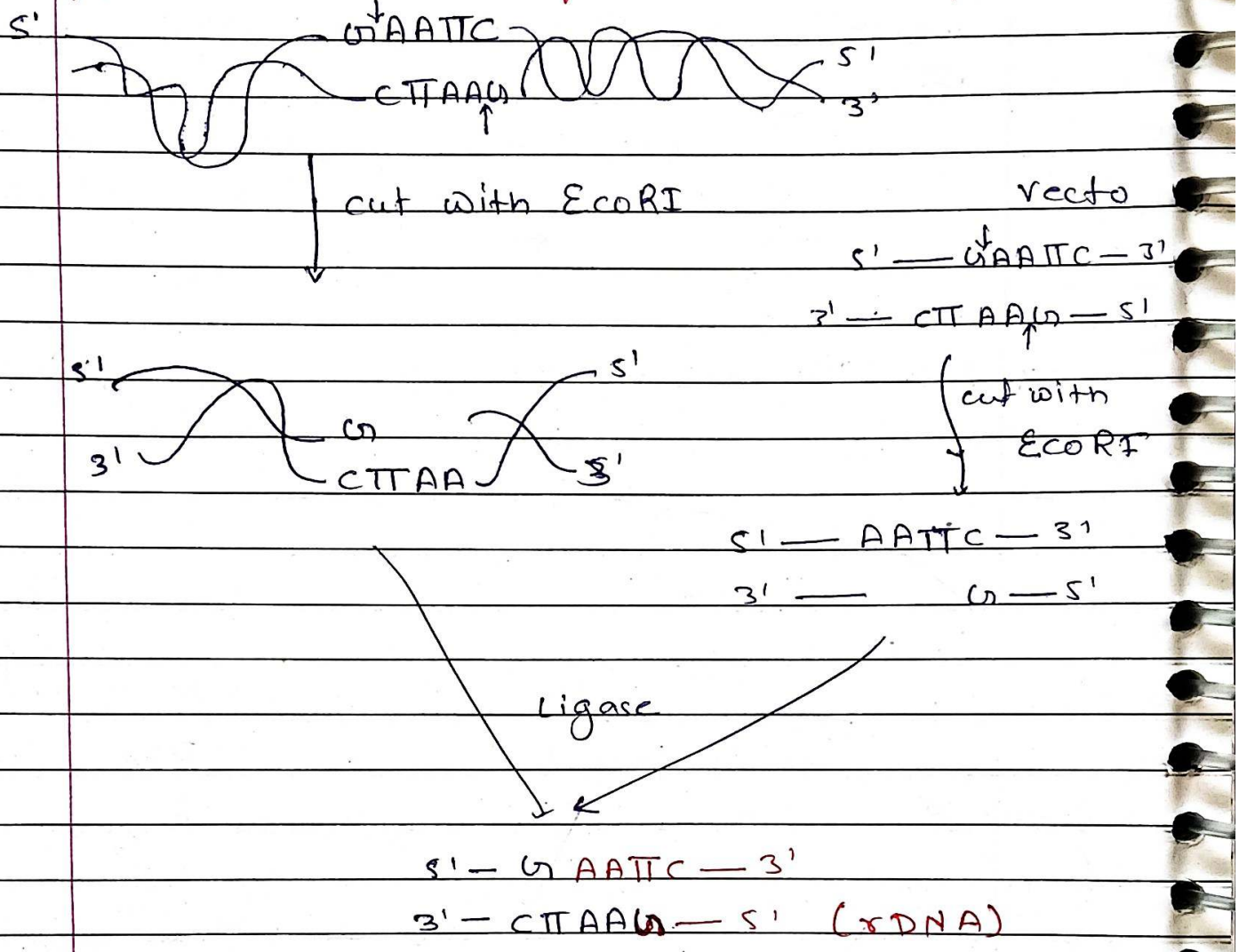
- Action mechanism of Restriction enzyme—



- Blunt end produce by ÷ EcoRV, HinII
- Sticky end produce by ÷ EcoRI, SalI, BamHI

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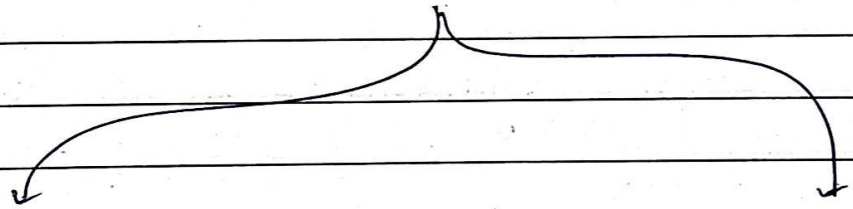
(3) ligase

→ Enzyme that perfor ligation
→ form phosphodiester bond b/w 2 adjacent nucleotides
→ Energy dependent process
→ known as genetic glue or gum

(4) ~~Poly~~ polymerase Enzyme

→ polymerase enzyme join nucleotides to form complementary DNA strand

→ DNA polymerase has two 2 activity



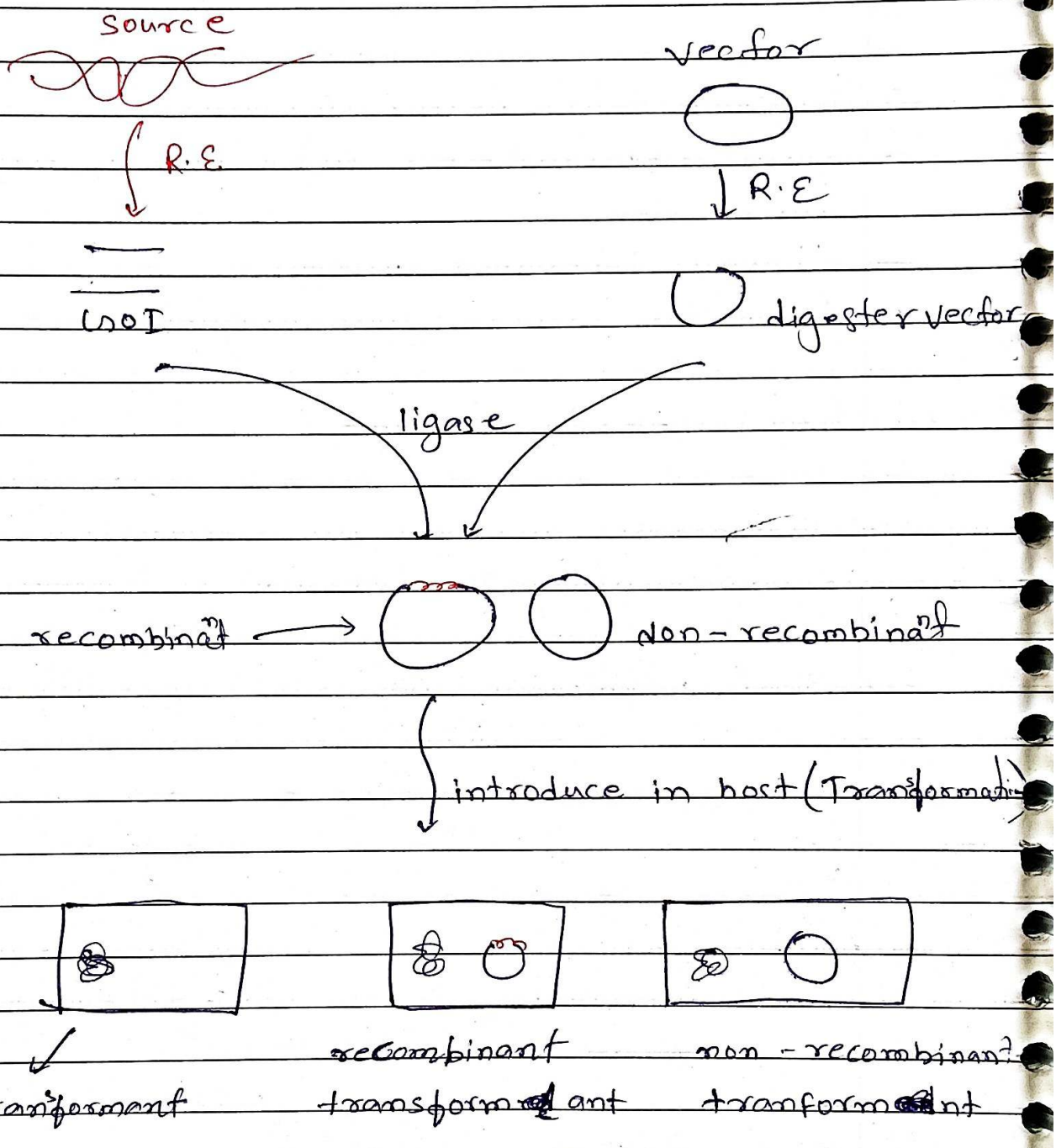
(a) polymerization (5'→3' direction)

(b) proof-read (check) (3'→5' direction)

(5) Cloning vector

→ Carrier of DOT that can be clone (multiplied)
→ It can be plasmid, Bacteriophage, YAC, BAC

→ We always use cloning vector with high copy numbers.
more copy no. → more multiplication


source
R.E
vector
R.E
digested vector
ligase
recombinant
non-recombinant
introduce in host (Transformation)
non-transformant
recombinant transformant
non-recombinant transformant

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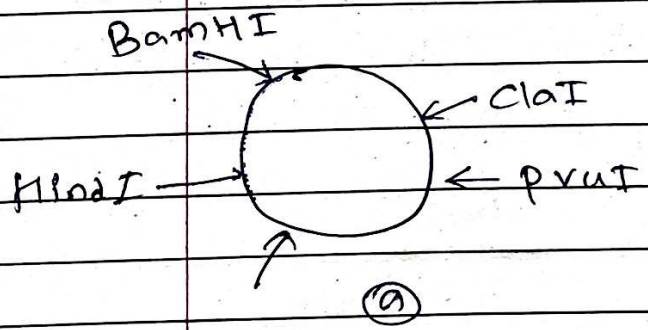
- Characteristics of cloning vector

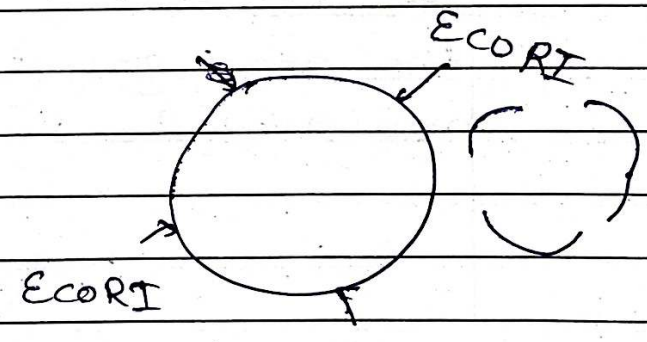
(a) Ori
→ Ori stands for origin of replication.
→ Sequence of DNA from where replication start
→ Ori support copy number.

(b) Rop
→ Sequence of DNA ~~where~~ which code for protein essential for replication

(c) Cloning site
→ Site where restriction enzyme cut
→ Vector with cloning site of different restriction enzyme is selected.
→ Vector with many cloning site of single restriction enzyme is not selected (result in fragments)



(a)
Choose



(b)
Not choose

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(4) Selectable marker

→ Property of vector which help us in - identifying (selection) transformant and non-tranformant or recombinant and non-recombinant

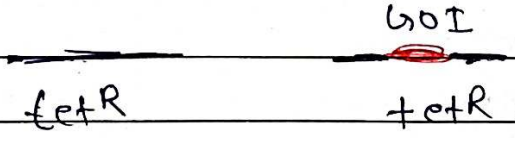
→ Mostly selectable marker are antibiotic resistance gene

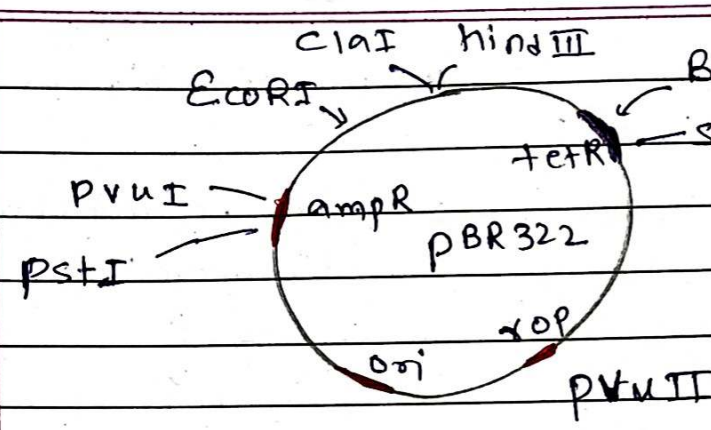
→ Normally E. coli lack resistance against antibiotic

e.g. ampicillin resistance
tetracycline resistance
Chloramphenicol resistance
Kanamycin resistance

- insertional inactivation

→ When we insert our GOI in antibiotic resistance gene (selectable mark), then the gene get inactivated due to insertion and antibiotic property is lost.

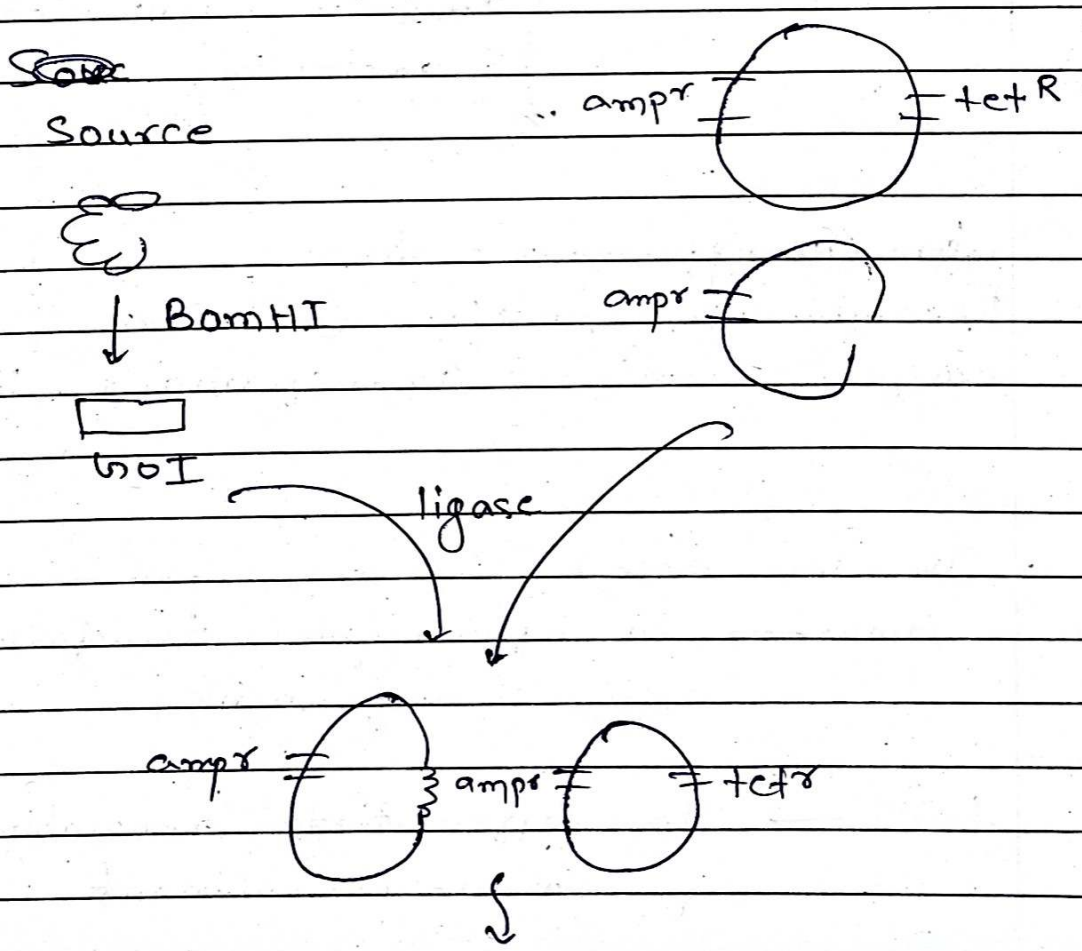




→ 8 cloning site
→ 2 selectable marker

.. p = plasmid
B = Bolivar
R = Rodriguez
322 = no. of experim

Note :- BamHI, SalI present in tetR
PvuI, PstI present in ampr



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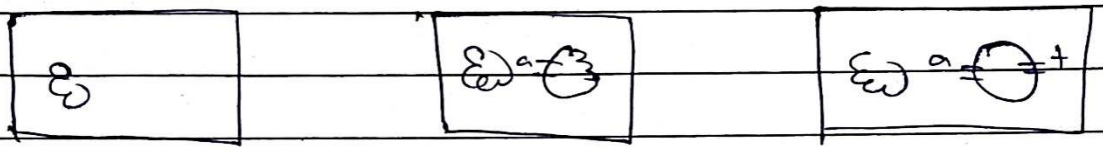
recombinant
non-recombinant

~~non-recombinant~~

grow in medium containing tetracycline

Transformation

non-recombinant live and recombinant die



Non-Transformant

recombinant Transformant

Non-recombinant Transformant

grow in medium containing ampicillin

Non-Transformant = die
Recombinant Transformant = live
Non-recombinant Transformant = live

- In this case,
  Amp^R ⟶ to differentiate transformant & non-transformant

  tet^R ⟶ to differentiate recombinant & non-recombinant

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	tet^r	amp^r
Recomb – Transformant	✓	✓
Non-recomb – transformant	x	✓
Non-transformant	✓	x
	x	x

- lacZ gene as Selectable marker

→ Above mentioned process, require 2 plating (+tetracycline and ampicillin) which is a tough process.

→ Scientists start using gene as selectable marker that codes for protein which convert substrate into color product.

→ Normally,

lacZ gen
↓ encodes
B-galactosidase enzyme

Xgal (substrate) → blue color product

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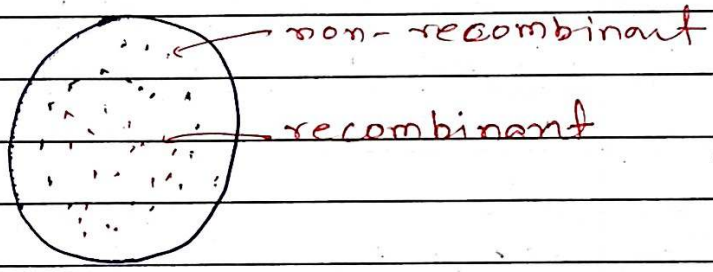
EcoRI

→ EcoRI inserted in lacZ gene, lacZ get inactivated, NO β-galactosidase, NO conversion of X-gal. White color appear

- In this, we get 2 color colony :-
  (i) Blue color → Non-recombinant
  (ii) White color → Recombinant

This is known as Blue-White color screening



- Other cloning vectors

(i) Bacteriophage :- Virus that attacks on bacteria
(ii) plasmid :- (important pBR322)
(iii) BAC :- Bacterial artificial chromosome.
(cc = upto 350 Kb)
(iv) YAC :- Yeast artificial chromosome (cc = 1 mb)
(v) Cosmid (cos site + plasmid) (cc = 30-45 Kbp
(vi) phagemid (bacteriophage + plasmid) (cc = upto 10 Kb)

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- Carrying capacity (cc)

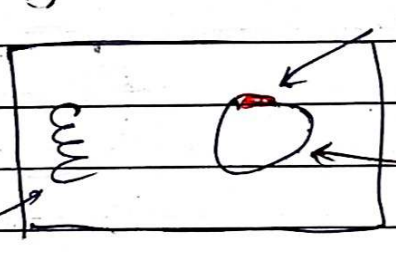
→ Length of GOI that can be carried by vector

- Cloning vector for plant

→ Agrobacterium tumifacien is a pathogen for several dicot plant

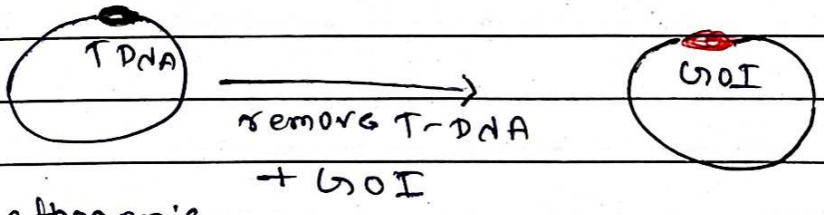
→ It has Ti plasmid (tumor inducing) which has T-DNA (transfer DNA)

→ When T-DNA get incorporated in plant's genome it cause tumor or node, known as Crown gall disease



Agrobacterium tumifacien

- (disarm) modified Ti plasmid is used as vecto in plant.



pathogenic Ti plasmid

disarm Ti plasmid

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- Cloning vector for animal

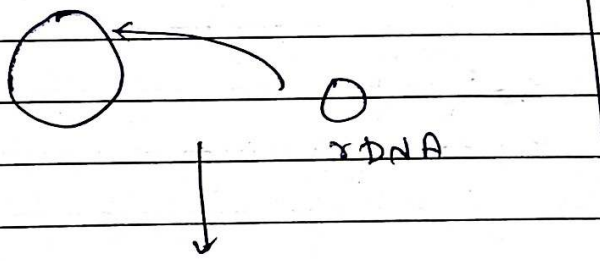
→ Retrovirus are disarm and then used as cloning (- pathogen gene) vector in animal

⑥ Competent host

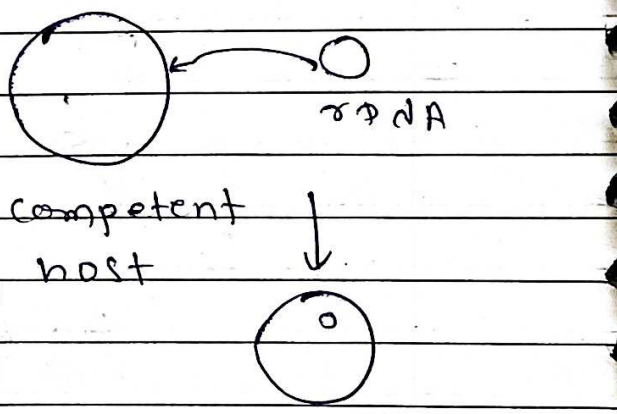
→ Compentent hosts are those hosts that is ready to take foreign DNA

→ Compentent host is ready for transformation

→ To make host competent, we treat them with divalent cation(Ca^{2+})



No tranformation as host is not competent



Competent host accept rDNA

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- Method of transformation

(1) Heat shock treatment

Competent host + rDNA (incubate)

↓

ice

↓

42°C

↓

ice

Sudden change in tem. cause formation of pores in cell membrane of host enabling entry of rDNA

(2) Gene-gun or Biolistic
→ for plant
→ micro-particle of tungsten and gold coated with rDNA is bombarded on plant cell with high velocity.

(3) Micro-injection
→ Needles carrying rDNA deliver rDNA directly in nucleus of animal cell.

(4) Electroporation
→ Use of voltage to create pores in cell-membrane of host to enable entry of rDNA

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(v) Disarmed vector
→ e.g. Ti plasmid for plant
retrovirus for animal

Note:- DNA is hydrophilic
, cell mem. interior hydrophobic (that's why DNA can not enter in cell directly.

Gel Electrophoresis

- purpose
  ↳ Seperate DNA fragments (basis - their size)

→ DNA is -ve charged (due to phosphate), forced to move toward +ve terminal (anode) in an electric field on the basis of their size

→ Smaller fragments move fast (away from cathode) & larger fragments move slow

→ Matrix is made of agarose (natural polymer) (exctracted from sea weed)

→ Ethidium bromide (Etbr) is used as stain that is used for visualisation of DNA fragment

→ Under UV ray, visualisation is done

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→ Bright organ color bands are observed under UV-light

→ Desired DNA fragment is cut out of gel, and this process is known as Elution process.

Note:-

↳ Ethidium bromide is carcinogenic
↳ Combs are used to prepare walls
↳ In wells DNA sample is loaded
↳ Ladder is used as reference for size of DNA

• Steps in Gel Electrophoresis

(1) prepare gel (with the help of agarose)

(2) Cast gel in tray (use comb to make wells)

(3) Rest the gel for solidification

(4) Remove comb (wells are formed at cathode site)

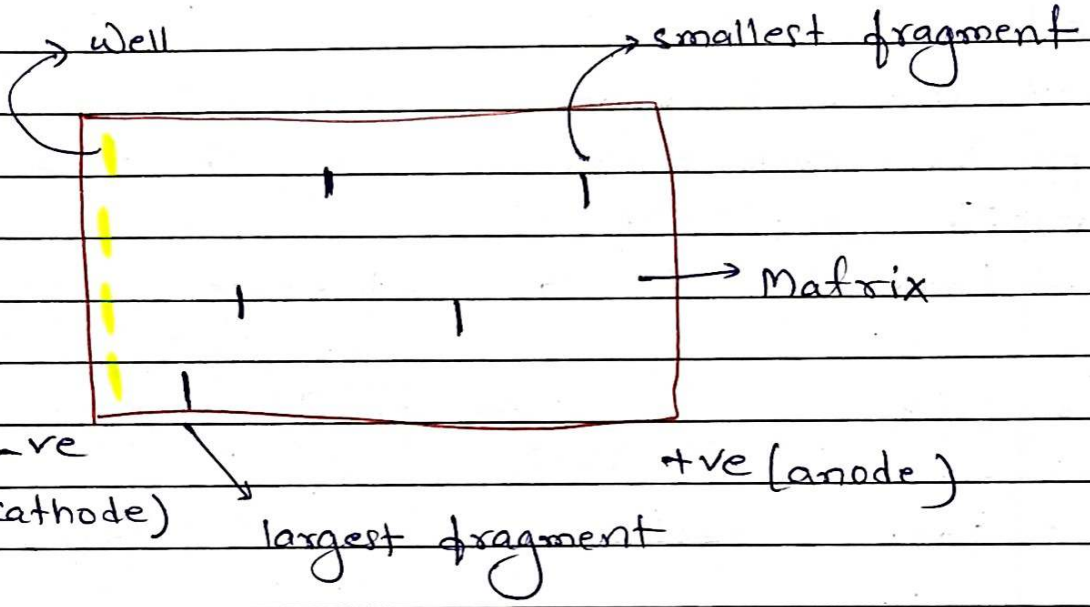
(5) Load DNA is wells

(6) Switch on the power supply

(7) DNA fragments move towards anode (small = fast, large = slow)

(8) Observe the DNA under UV rays.

Note:- DNA fragments are seprated through — sieving effect.



(Gel Electrophoresis)

* PCR

→ PCR stands for polymerase chain reaction
→ Give by Kary Mullis in 1983
→ ATM:- In vitro replication, make multiple copies of DNA fragment (DOI)

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- Requirements for PCR :-

(i) Tempelate DNA
(ii) DNA polymerase
(iii) Primers
(iv) dNTP
(v) Mg^{2+}, buffer

- 3 steps in PCR
(i) Denaturation
(ii) Annealing
(iii) Extension

(i) Denaturation

→ Ds DNA is seperated at high temperature (95°C)
→ H-bonds b/w nitrogen base pairs are broken (A=T) (G≡C)

(ii) Annealing

→ ~~forrm~~ forward and reverse primers are joined at both strand of DNA

→ Annealing temperature = 40-65°C

→ primers.
↳ Oligonucleotide sequence provided, which give free 3'OH for extension process

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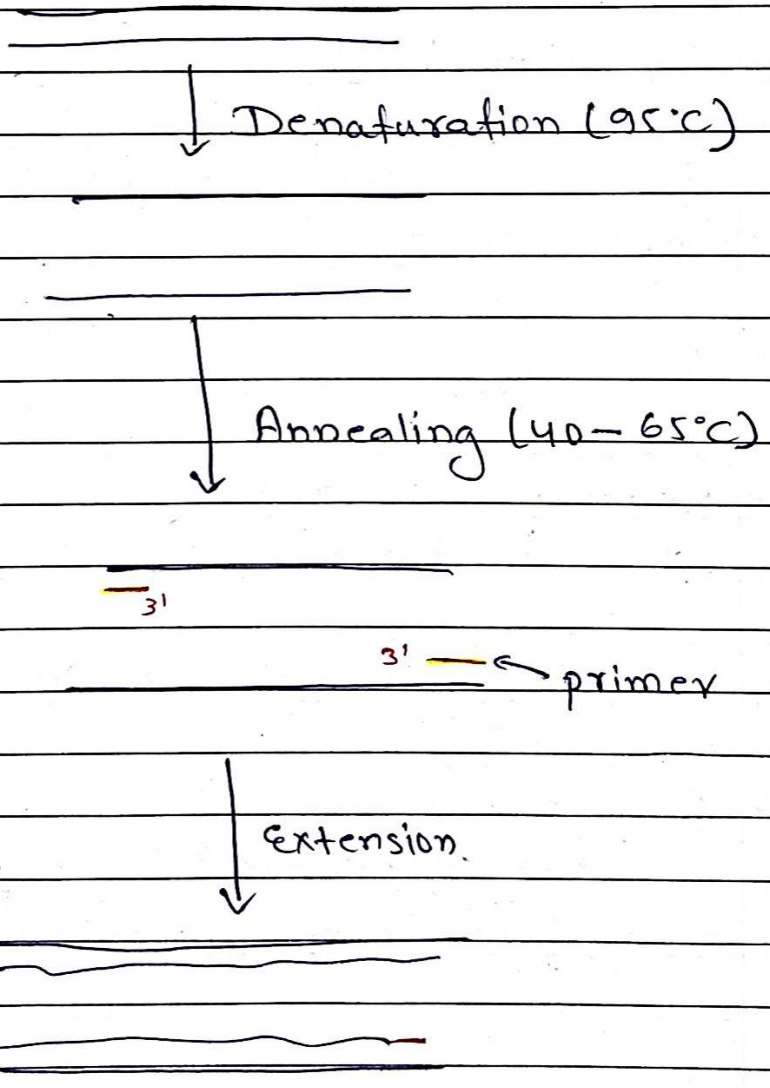
(3) Extension

→ DNA polymerase polymerise the complementary sequence (at 72°C)

→ Taq polymerase is used.

- thermostable
- Active at high temperature
- obtained from Thermus aquaticus



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Note:-

(i) No. of copies of DNA = 2^n (n = no. of cycle)

(ii) After 30 cycles 1 billion copies of DNA obtained

• Application of PCR :-

(i) DNA fingerprinting (ii) forensic science
(iii) Dectection of pathogen (iv) Dectection of mutated gene (v) prenatal diagonsis (vi) gene therapy

* Steps in recombinant biotech

(1) Isolation of DNA

→ DNA is genetic material in most of organism
→ DNA is present inside cell, So to obtain DNA we have to break cell

Component	Treatment
Cell wall ⟶	lysozyme (bacteria), cellulose, pectinase & chitinase (plant) & (fungi)
Cell mem. ⟶	detergen/lipase
RNA ⟶	Ribonuclease
protein ⟶	protease
Carbohydrate ⟶	Amylase
Lipid ⟶	lipase

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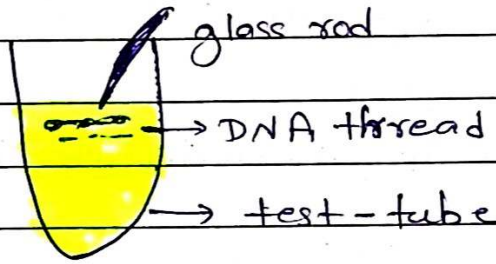
Note: No use of DNase because We Want to isolate DNA

→ We will precipitate DNA with the help of chilled Ethanol.

→ With the help of glassrod we will take out DNA thread, this process is known as Spooling



Spooling

(2) Cut GOI and Vector

→ Cut at specific location in source DNA and Vector DNA with same Restriction enzyme (molecular scissor)

→ DNA fragments seprated on Gel Electrophoresis

→ Both GOI and digested vector is isolated.

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(3) Make multiple copies of GOI (By PCR)

(4) ligation of GOI and Vector with the help of ligase (Genetic glue/gum)

(5) Insert rDNA into host (Transformation)

(6) Select our recombinant transformant with the help of selectable marker

(7) Produce at large scale

(8) Downstream processing

* Bioreactor

→ To produce product at large scale, bioreactor are used.

→ Bioreactors are large vessel in which 100 – 1000L culture is prepared.

→ Raw materials are provided & optimal conditions are given so that good quality product is produce.

→ Continous stirred bioreactors are most commonly used.

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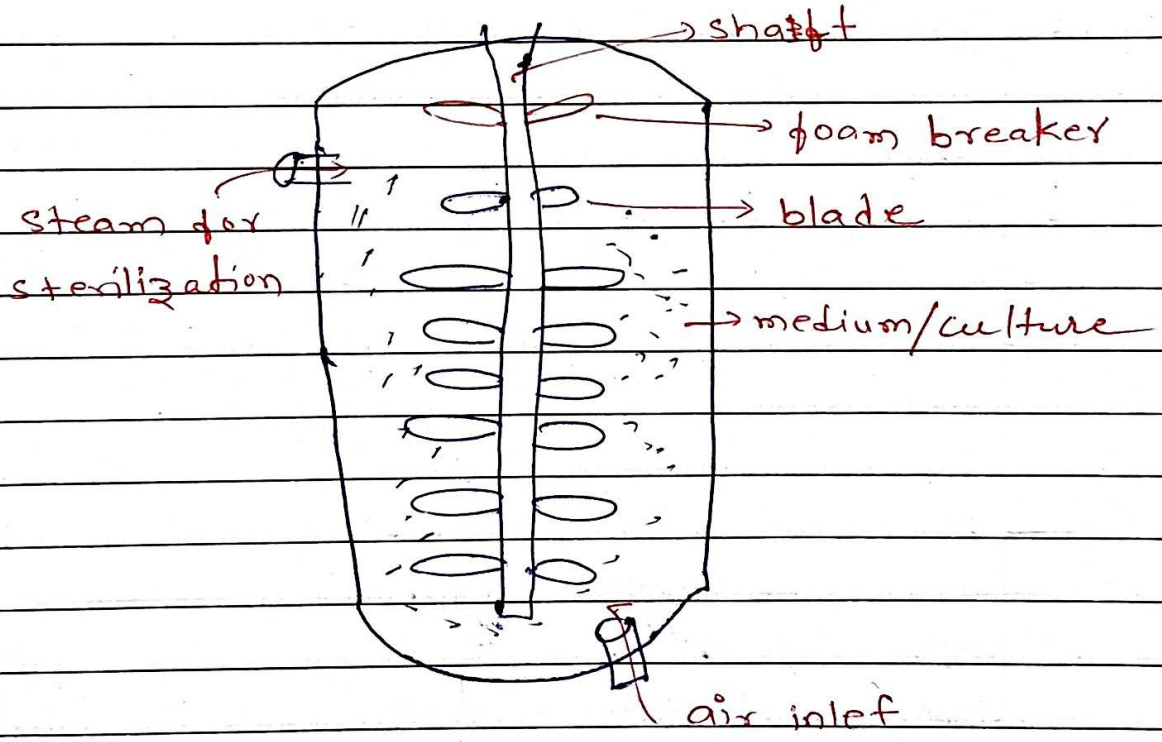
Types of bioreactor

- Simple - stirred bioreactor
- Sparged bioreactor
  - gas is provided in the form of bubbles to increase efficiency



Bioreactor

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Component of bioreactor

(i) Vessel (cylindrical or curved base)
(ii) Sensors for tem. & pH
(iii) Agitator system (motor, shaft, impeller for homogenous mixing)
(iv) Gas/oxygen delivery system
(v) Foam control/ braker
(vi) Temperature control
(vii) pH control

* Continous culture system

→ Open type
→ used medium is drained out from one side
→ fresh medium is added from other side
→ most active log phase
→ high yields.

* Batch culture system

→ closed type
→ One time medium addition and output recovered.
→ log phase is short.

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* Downstream processing

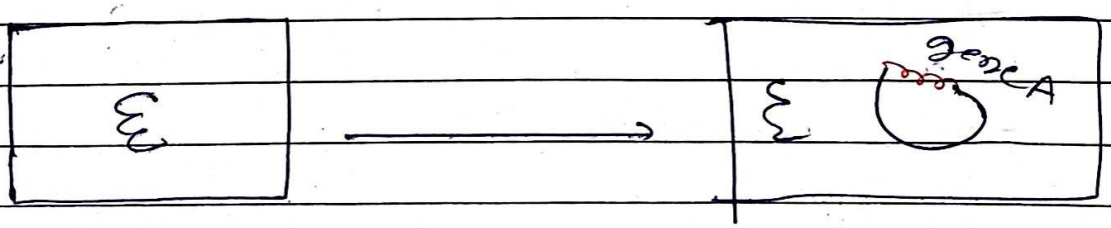
→ Includes steps after production of products
→ like :- seperation, purification, addition of preservative, Clinical trails (drugs), Quality check

ote :- Different product has different downstream process.

* Heterologus protein

→ protein enceding gene expressed in heterologus host.

- Heterologus host
  - → Host in which naturally that protein is absent
  - → When we insert rDNA (having that gene) then host express that protein



bacteria
enzyme 'A' absent

bacteria
enzyme 'A' present
↓
protein A heterologous
Bacteria hetero-logous.

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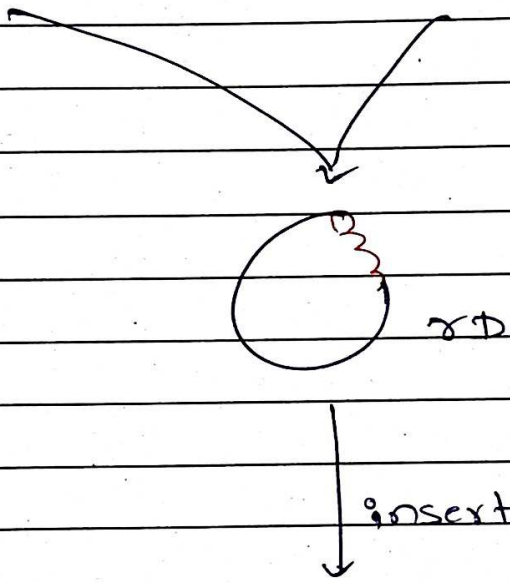
★ Construction of 1st rDNA

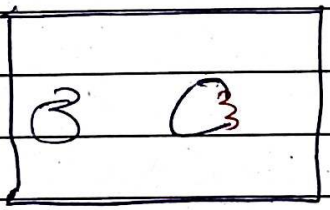
→ By Stanely Cohen & Herbert Boyer (1972)

→ ab^r + plasmid (Salmonella typhi)



rDNA

inserted in E. coli



↳ show ab^r property

Biotech and its Application

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* Introduction

Application of Biotech (majorly)

① Agriculture
↳ Use of 'Genetically modified crop'

② Medicine
→ Biopharmaceutics (drugs/medicine prepared using GMO)
→ Biotherapeutics (use for therapy & treatment of Diseases)
→ Diagnosis (PCR, ELISA)

③ Waste treatment
④ Bioremediation (use of living organism for treatment of H_2O/ soil/ Air pollution)
⑤ Energy production. (Biogas)

* Three critical research area in biotech

① finding the best catalyst in the form of improved organism which is a microbe or pure enzyme

② Creating ~~optium~~ optimum conditions for catalyst to act

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③ Down stream processing

* ~~Biotechno~~ Application of biotech in Agriculture

There are three ways to increase food production

① Use of harmful chemical (Agro-chemical based Agriculture)
e.g. fertilizers, pesticides

② Organic farming: Biofertilizers, Biopesticide
e.g. N_2 fixing bacteria

③ Use of GMC

- Green Revolution

→ Norman E. Borlaug → father of green revolution (globally)

→ M.S. Swaminathan → father of green revolution in India

→ Because of green revolution in India food production increase 3 times but wasn't enough for ~~po~~ increasing ~~popult~~ population.

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→ Food production increase 3 times due to :-

(1) Better management practices (Bette irrigation, machines, croprotation)

(2) Used of fertilisers, pesticides

(3) Improved Cropvarieties

* Genetically modified Crops :-

(1) Can tolerate any abiotic stress (Tem/pH/salinity etc)

(2) ↑ productivity

(3) Reduced reliance (dependency) on pesticides, fertilisers.

(4) Make pest - resistant plant (Bt - cotton)

(5) Better or ↑ Mineral usage from soil by plant

(6) Reduced post - harvest loss
e.g. ~~flower~~ flaver savr Tomato
↳ Gene silencing used

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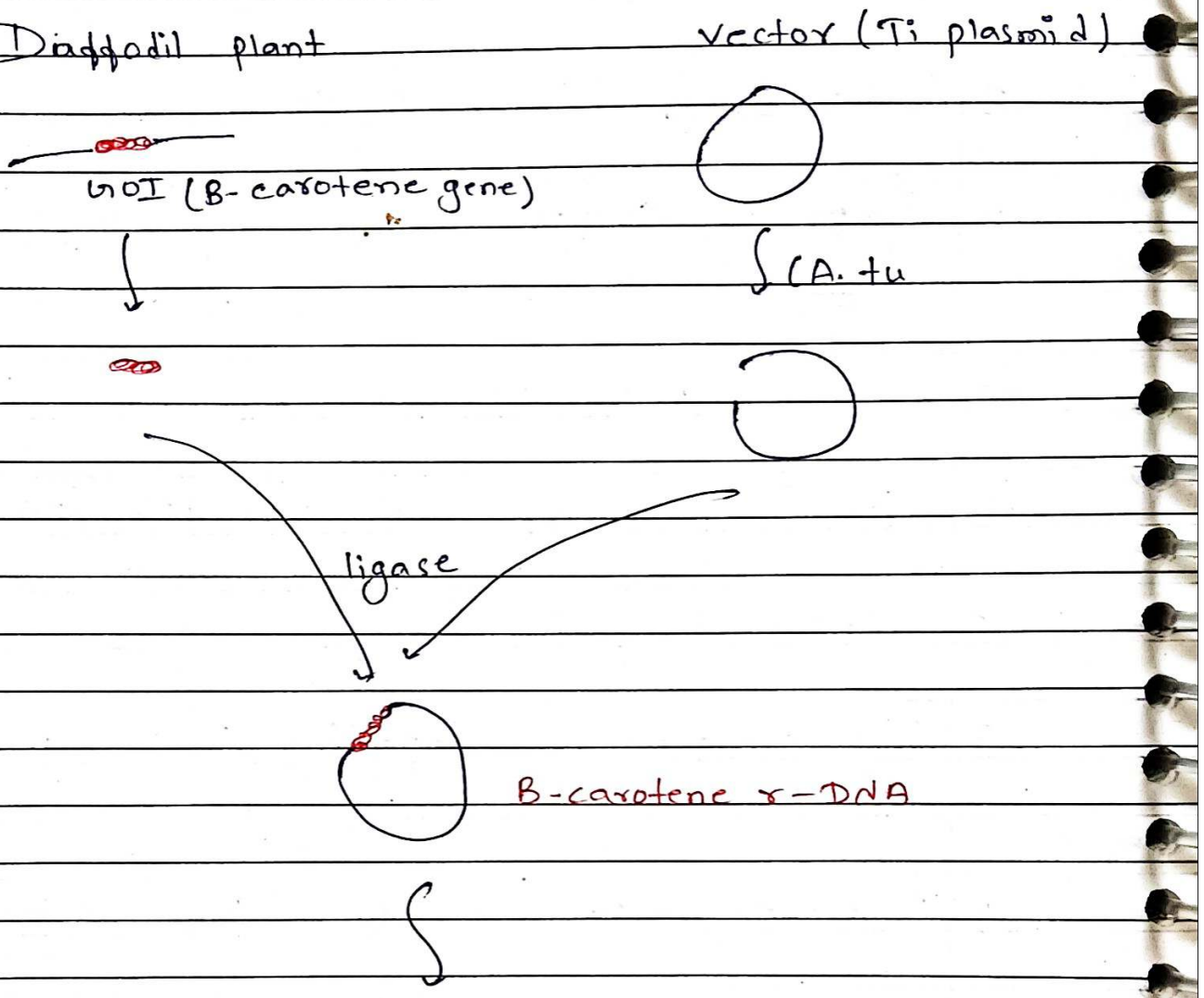
⑦ Enhanced Nutritional value

e.g. Golden Rice (vit-A enrichment)



Inserted in normal rice grain variety (plant)

synthesise/ form

B- carotene

Eventually form vit-A

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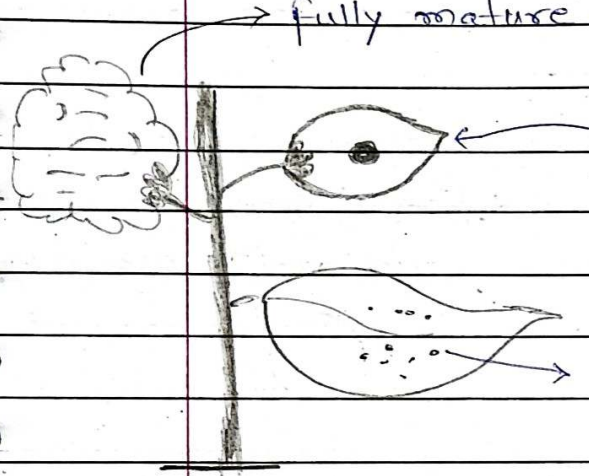
Bt - cotton (pest - resistant plant)



↓

found occasionally on cotton plants

↓

It was observed that if 'cotton' - Bollworms (insect) feeds on this plant & eats the bacteria

↓

It dies

Bacillus thuringiensis

↓

'cry' gene

↓

At particular phase of growth, cry gene form cry protein

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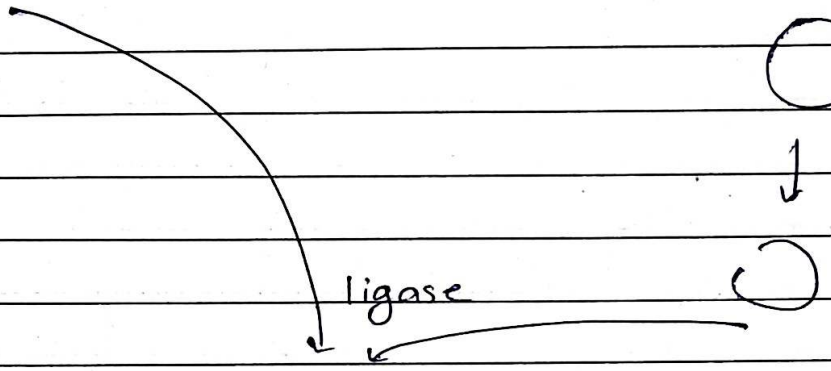
- Cry protein
  - → Endo-toxic
  - → protoxin

cry gene

~~Bt~~ – cotton

vector (Ti plasmid)



r-DNA

↓

inserted in cotton plants

↓

cry gene

↓

cry protein (inactive)

↓

Insect (cotton ballworm) eats plant

↓

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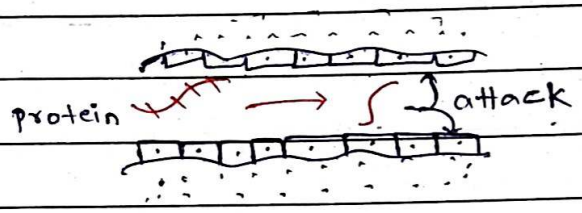
Cry protein enters inside insect ~~body~~



epithelial cells of midgut of insect

- In the alkaline pH of midgut these protein get activated

Causes pores in Epithelial cell

Fluid from surrounding enters cell

Cell swells & burst

insect die

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- Some important points

(1) Choice of cry gene depends on → The pest & crop

(2) Cry I Ab → Corn borer

(3) Cry II Ab, cry I Ac → cotton bollworm

Extra

(3) phylum Arthropoda

↓

class Insecta

↓

Orders

→ Lepidopterens : Tobacobudworm, Armyworm, Cotton bollworm

→ Coleopterens: Beetles

→ Dipterens: flies, Mosquito

Cry protein is toxic

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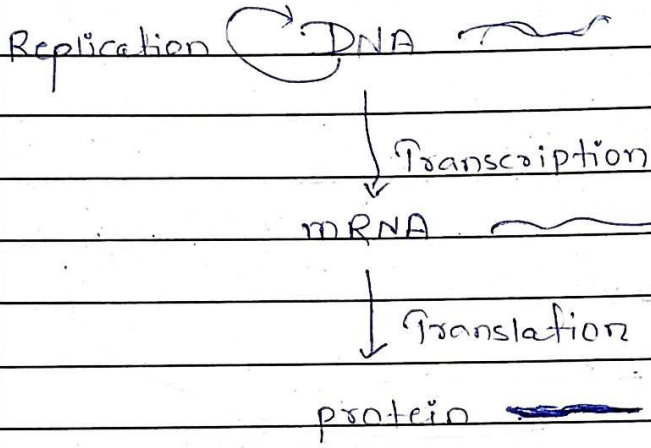
✱ RNAi / m-RNA silencing
↳ RNA interference



- In gene silencing : mRNA needs to be silent
  ↳ Done by making dsRNA
  ↓
  No protein

Note:-

(i) RNAi takes place in all eukaryotes as a mechanism of natural cellular defense

(ii) Here, the source of complimentary mRNA is through infection of RNA virus. or Transposons (Jumping gene) that replicate via RNA intermediate.

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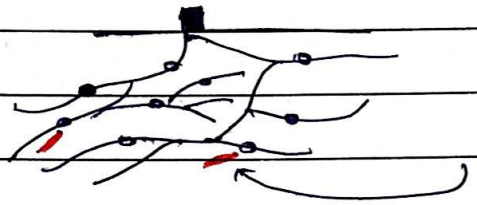
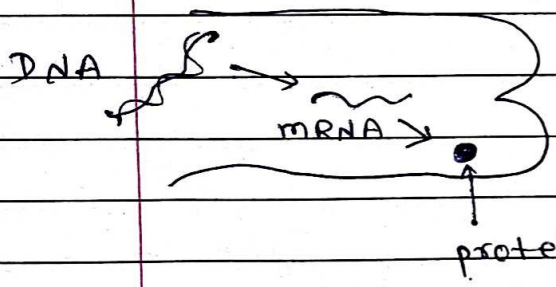
- Tobacco plants (pest - resistant)



Nematode
↳ Meloidegyne incognitia

infected tobacco plant
(root - knot disease)

cause



Nematode ↳ essential for feeding & survival

- Our motive stop translation of this specific protein. So that the nematode does not grow

↳ for this we will stop translation

↳ We will convert ssRNA into dsRNA, done by RNA interference / RNAi / mRNA silencing / anti-sense technology

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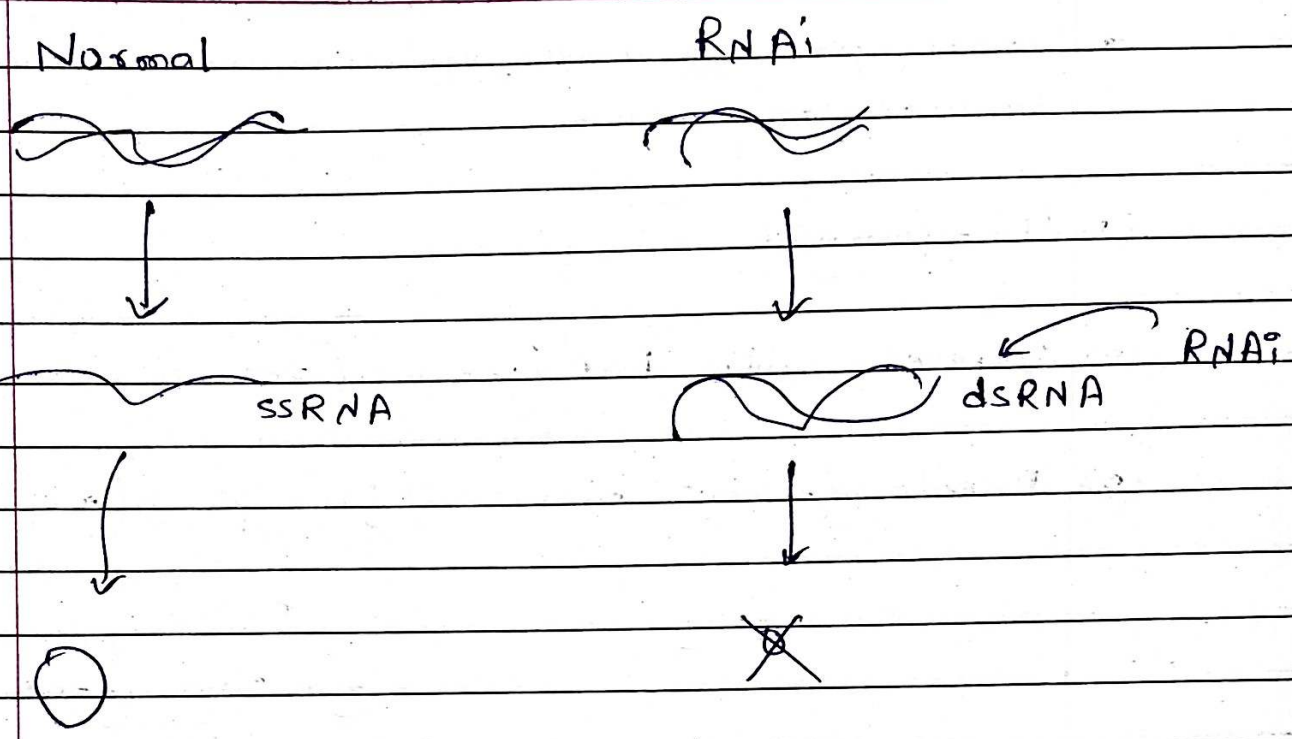
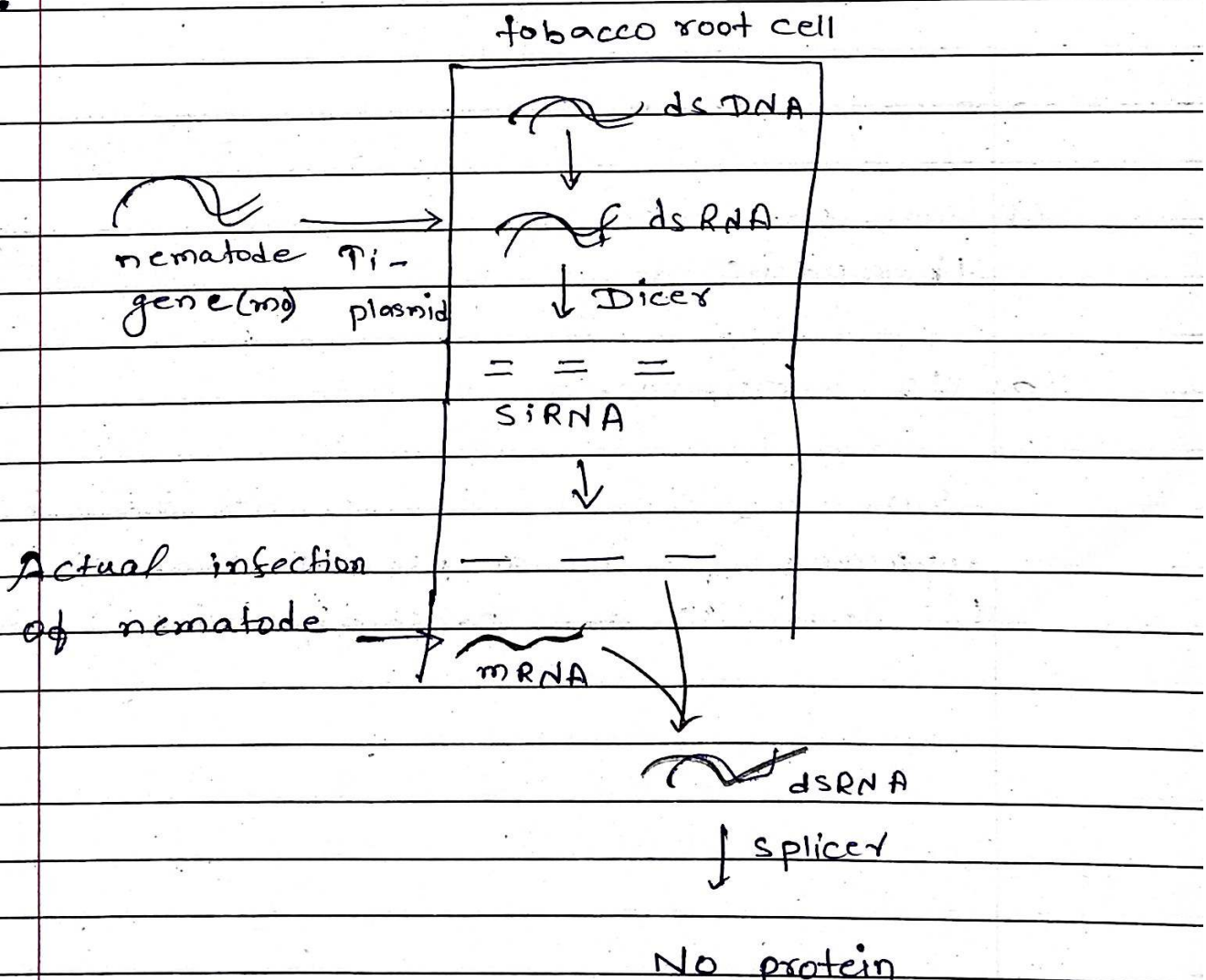

Normal
RNAi
ssRNA
dsRNA
RNAi


tobacco root cell
dsDNA
dsRNA
Dicer
siRNA
nematode Ti-gene(mg) plasmid
Actual infection of nematode
mRNA
dsRNA
splicer
No protein

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- There are 3 ways to introduce rDT in tobacco root cell

(1) Ti-plasmid

(2) Viruses with RNA genome (retro-virus)

(3) Mobile genetic elements/ transposon (replicate via RNA)

"jumping gene"

* Application in Medicines

- Role of rDNA technology in medicine:

(1) Mass production of safe and more effective therapeutic drugs

(2) No immunological response

Note:- 30 recombinant therapeutics have been approved for human use all over the world. In India 12 are presently being marketed

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Diabetes

Diabetes mellitus

B-cell of pancreas

↓ secrete less

insulin

insulin ↓ , sugar in blood ↑

Diabetes insipidus

↳ vasopressin ↓

water reabsorbit ↓

urination ↑

- Insulin

→ In case of diabetes(D.M) patient take insulin from outside as the beta cells of pancreas donot produce enough insulin and the level of sugar is high.

→ Earlier insulin was isolated from pancreas of slaughtered cattle and pigs

↳ Disadvantage :- Cause allergy or other reaction

↳ Solution :- prepare insulin in lab with the help of rDNA technology

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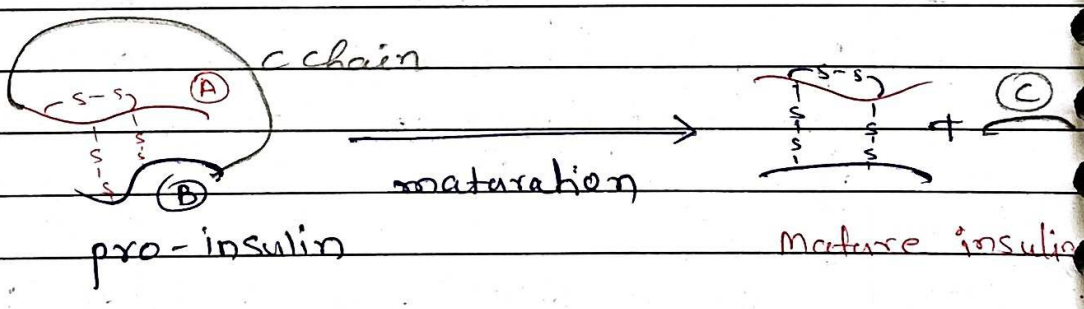
- Structure of insulin:

Insulin gene
↓

proinsulin (inactive)
↳ chain A(21aa) and chain B(30aa) are connected by chain C(31aa)

→ A and B chain are connected by 2 disulphide bond. Also one disulphide bond is present within A chain

→ During activation of pro-insulin, C chain is removed and mature (functional) insulin is formed



A & B = 2 disulphide bond
Within A = 1 disulphide bond

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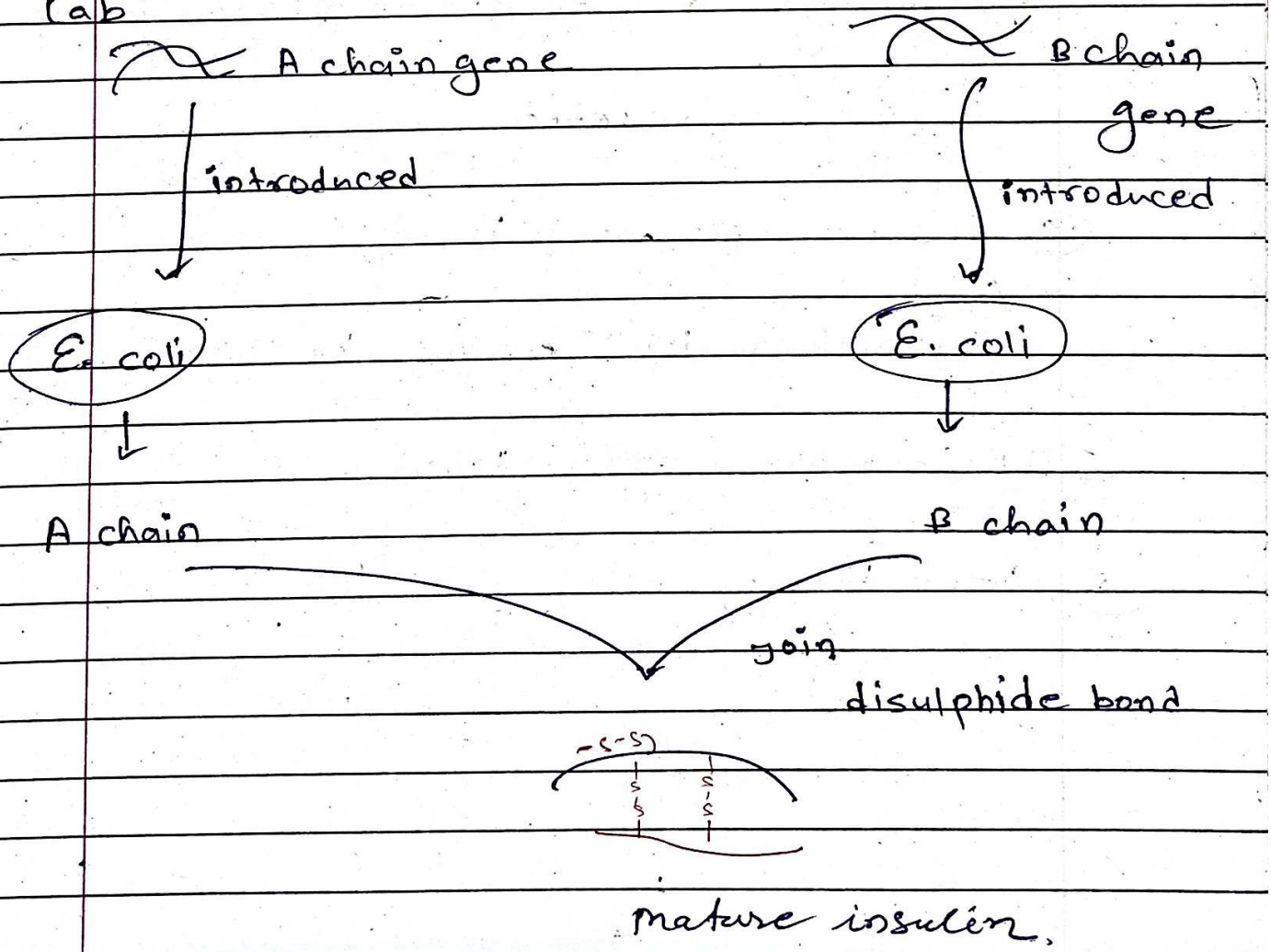
- But we can't produce genetically engineered insulin using E. coli because maturation or assembling of insulin is task

- In 1983 American company Eli lilly prepared synthetic gene for A chain and B chain. These gene were introduced in E. coli seprately. As a result A chain and B chain produced separately. At the end these 2 joins were joined by forming disulphide bonds

↳ In this way Humulin was produced (1st GE insulin/hormone)



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Gene therapy

→ It is collection of methods that help us to correct gene defect that has been diagnosed in child/embryo.

→ Here, normal gene is delivered to the individual to replace non-functional gene.

→ 1st time gene therapy was given to 4 year girl in 1990. This girl was suffering from SCID – ADA (severe combined immuno deficiency / Adenosine deaminase enzyme).

→ ADA (by ADA gene) is necessary for immune functioning as well as lymphocyte development.

Treatment of defect gene

(1) Enzyme replacement therapy
↳ functional ADA enzyme by injection

(2) Bone marrow transplant

(3) Gene therapy

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- Gene therapy

Take blood from patient

↓

Separate lymphocyte

↓

Culture lymphocyte

↓

Introduce functional ADA (c-DNA) into lymphocyte, using retrovirus.

↓

6 lymphocytes returned back into patient's blood

- ADA gene isolate (ds)

↓

ADA RNA(ss)

↓

This ss RNA is ligated with disarmed retrovirus's genome (vector, RNA)

↓

This vector is introduced in host (lymphocyte) and deliver ss ADA RNA in lymphocyte

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↓

ss RNA —reverse transcription→ CDNA

↓

CDNA integrated in lymphocyte DNA and codes for functional ADA enzyme.

→ Not complete cure; as lymphocytes have lifespan (not immortal)

→ priodic infusions of bE lymphocytes.

permanent cure for SCID-ADA:

↳ If gene therapy given at early embryonic stage (totipotent stem cell)

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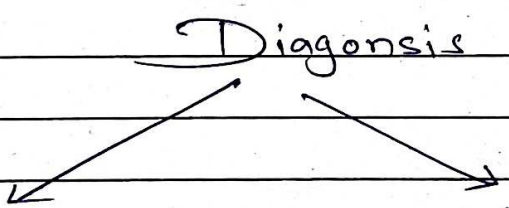
Molecular diagnosis

↳ Early diagnosis help us for effective treatment
↳ When symptoms appear, the conc of pathogen is high
↳ At low concentration of pathogen, no symptoms appear.

- Conventional diagnosis
  ↳ serum analysis, blood test, urine test etc
  ↳ Early detection not possible

↳ for early ~~diagnosis~~ → detection ÷ rDNA tech, PCR, ELISA



Early
↳ symptom ⊖
PCR, ELISA
↳ pathogen concentration ↓

late
↳ symptom ⊕
↳ conventional diagnosis technique
↳ pathogen concentration high

- Window period
  ↳ Time period b/w infection and symptoms appearness.

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→ In PCR, pathogen specific primers are added If multiplication occur meaning the pathogen is present

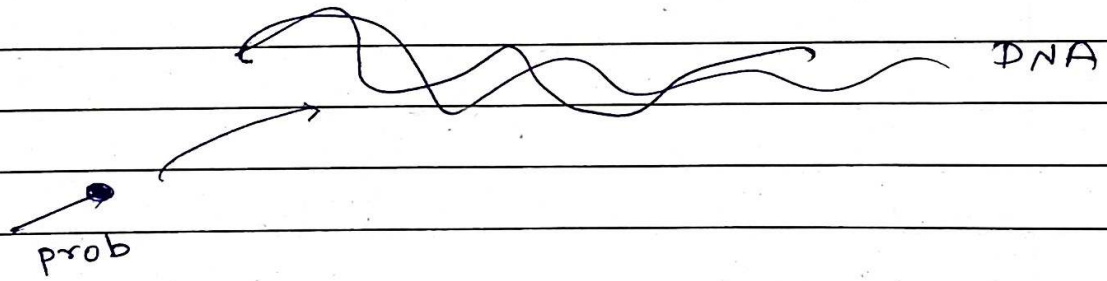
→ PCR is used in detecting HIV, to dectect mutation in gene, detect genetic disorder.

* Autoradiography

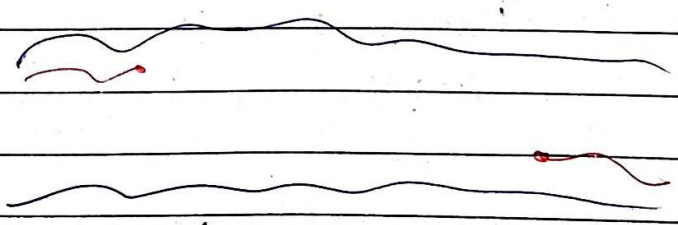
↳ To detect mutation in gene in a individual

CASE I

Normal DNA gene (no mutation)



- prob
  ↳ ssDNA/RNA complementary to normal gene tagged with radioactive material



↳ x-ray (light appear)

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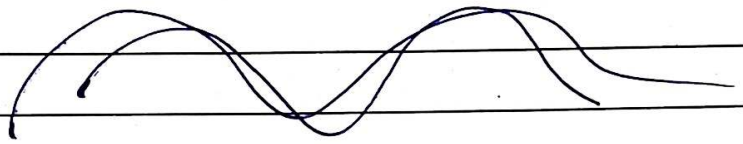
CASE II (mutated gene)

↳ Mutation



probe

↓

not bind

↓

not light

↓

Mutation

* ELISA

↳ enzyme linked immuno sorbent assay)

→